International Journal of Botany
and Research (IJBR)
ISSN (P): 2277-4815; ISSN (E): 2319-4456
Vol. 7, Issue 5, Oct 2017, 1-8

TRANS STELLAR
•Journal Publications •Research Consultancy

EVALUATION OF OPTIMUM CONDITION FOR REDUCING SUGAR PRODUCTION FROM *EICHHORNIA CRASSIPES* BIOMASS BY SACCHARIFICATION THROUGH *TRICHODERMA REESEI*

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ABSTACT

Water Hyacinth is used as renewable energy raw material for sugar production and then fuel production because, its availability is easy and rate of propagation is very high. It is an aquatic weed, tolerates variation in pH and temperature and is unaffected by toxic substances. Determination of reducing sugar from lignocellulosic biomass of Water Hyacinth was carried out by saccharification process, using micro fungi as trichoderma reesei. It was achieved using dinitro salicyclic assays (DNS method). Reducing sugar contents were evaluated maximum at optimum period. The amount of sugar contents were maximum which were 547 µg/ml in untreated condition and 596 µg/ml in treated condition at optimum inoculation period these were found after inoculation of seventh day and 365 µg/ml, 458 µg/ml and373 µg/ml in untreated condition and 521 µg/ml, 593 µg/ml and 492 µg/ml in treated condition at optimum conditions of pH, temperature and substrate concentration which were 5 or 5.5, 30^0C and 2.0 or 2.5 ml respectively. Here the two types of samples were prepared. One was treated with 1%H_2SO_4 and other was untreated. The reducing sugar contents were obtained more in treated condition in compression to untreated condition. The present study evaluated Water Hyacinth as raw material for possible strategies by conversion of hydrolysate to reducing sugar was maximized. For this the effect of optimum condition such as inoculation period, pH, temperature and substrate concentration on conversion of Water Hyacinth biomass to reducing sugar was studied.

KEYWORDS: Aquatic Weed, Saccharification, Hydrolysate, Optimization & DNS

Received: Jul 29, 2017; **Accepted**: Aug 18, 2017; **Published**: Sep 11, 2017; **Paper Id.**: IJBROCT20171

Original Article

INTRODUCTION

Water hyacinth plant is known as aquatic weed belonged to the monocotyledonous family Pontederiaceae (Alison, 2000). Several fungal pathogens have been reported to attack water hyacinth in various parts of the world (Alison, 2000; Butt *et, al*, 2001). Fungi are the main cellulose degrading microorganisms through cellulose production, though a few bacteria and actinomycetes have also been reported to yield cellulase activity. Fungal genera like *Trichoderma*, *Aspergillus* and *Fusarium* are known to be cellulase producers and crude enzymes produced by these microorganisms are commercially available for agricultural use. The genus *Aspergillus* species attack cellulose producing significant amount of cell free cellulase capable of hydrolyzing cellulose into fermentable soluble sugars such as glucose; an important raw material in chemical industries (Wainwright, 2010). *Aspergillus* and *Trichoderma* species are well known efficient producers of cellulases (Peij *et al*, 1998). Several studies by Mandels and Reese (1985) have been carried out to produce cellulolytic enzymes from bio waste degradation process by many microorganisms including fungi such as *Trichoderma*, *Penicillium* and *Aspergillus* species etc.

Cellulose is the most abundant component of plant biomass. It is found in nature almost exclusively in plant cell walls, although it is produced by some animals e.g, tunicates and few bacteria (Lynd *et al*, 2002). Any process which could efficiently and economically convert cellulolytic material to glucose would be of immense industrial significance (Walsh, 2002). Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation (Lederberg, 1992). However, fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd *et al*, 2002). Cellulase catalyzes the conversion of insoluble cellulose to simple, water soluble products (Alexander, 1961). This study aims to provide better understanding of optimum condition for the production of reducing sugar through saccharification by *Trichoderma reesei*.

MATERIALS AND METHODS

Sampling Collection

Fresh water hyacinth plants with long stem were collected from Laxmi Taal of Jhansi city. Collected water hyacinth sample were washed to remove adhering dirt and chopped in small pieces. These small pieces were dried in sunlight. Dried water hyacinth biomass was pre-treated 1% v/v H_2SO_4 with soaking time of five hrs at room temperature. Pre-treated samples washed neutrality with distilled water and then dried in hot air oven and powered in grinder and stored in dry place for further use. We found finally two sample (treated and untreated) for hydrolysis and fermentation. Untreated samples meant without 1% H_2SO_4. Pre-treatment is required to alter the biomass macroscopic and microscopic size and structure as well as its sub microscopic chemical composition so that the hydrolysis of carbohydrate fraction to monomeric sugar can be achieved more rapidly and with greater yield (Sun and Cheng 2002; Moiser *et al*, 2005). Hydrolyaste was prepared by mixing the dried power with 8 volume of 1%v/v sulphuric acid for 7 hours in a glass lined reactor stirred at 250 rpm on rotator shaker. The mixture was autoclaved at 121 °C, 15 lbs for 15 min and further cooled down at room temperature. The hydrolysate was filtered using Whatman filter paper No. 1 to remove the unhydrolysed material and wash with warm water (60^0C). The filtrate and washing were pooled together (Carvelheiro *et al*, 2008) and this hydrolysate was detoxified by Ca $(OH)_{2.}$ Then it was filtered to remove insoluble and filtrate was used for observing fermentable sugar.

Preparation of Media

For reducing sugar production, Mandel's media was used (Mandel and Weber1969). This media contained following ingredients: Ammonium sulphate -1.4 gm, potasium dihydrogen sulphate -2.0gm, Magnesium sulphate – 0.3 gm, Calcium chloride 0.3 gm, Ferrous sulphate -0.005 gm, Mangnese sulphate -0.0016 gm, Peptone -1.0 gm, Urea -0.3 gm, Zinc chloride -0.0017 gm, Cobaltous chloride – 0.002 gm, CMC sodium salt -10 gm. All above chemical dissolved in distilled water and make up to 1000 ml.

Preparation of Inoculums

Trichoderma reesei was grown on PDA slants at 27 ± 2^0C for 6 days and maintained as stock culture, then stored at 40C. Inoculum was prepared using potato dextrose broth in 250 ml conical flasks. Inoculums rotated at 250 rpm at 30^0C for 24 hours and then used for fermentation

Saccharification

Mandel's media was taken in conical flask and added treated and untreated hydrolysate separately. All flasks plugged with cotton wool and then, sterilized in autoclave at 121^0C and 15 lbs for 30 min. 1 ml of spore of Inoculums of

Trichoderma reesei was inoculated in each conical flask containing Mandel's media after cooling.

Effect of Inoculation Period

All flasks were kept in incubator at 30°C for different days as 3, 5, 7,10,13,16 days. All flasks were shaken twice daily. Now these samples were used for DNS test for total reducing sugar.

Effect of pH

Before autoclaving pH of Mandel's media containing hydrolysate were adjusted at various level of pH by adding 1 N NaOH and 1 N HCl solution. pH ranges were adjusted as 3.0, 4.0, 5.0, 5.5, 6.0, 6.5, and 7.0. All flasks were kept in incubator at 30°C for 7 days. All flasks were shaken twice daily. Now these samples were used for DNS test for total reducing sugar.

Effect of Temperature

All autoclaved flasks containing media with spore suspension and hydrolysate kept in incubator at different temperature ranges as 20^0C, 25^0C, 30^0C, 35^0C, and 40^0C for 7 days. All flasks were shaken twice daily. Now these samples were used for DNS test for total reducing sugar.

Effect of Substrate Concentration

Different amount of treated and untreated hydrolyate ranging from 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0 ml, 3.5 ml, 4.0 ml, 4.5 ml, 5.0ml were mixed with Mandel's media. All flasks plugged with cotton wool and then, sterilized in autoclave at 121^0C and 15 lbs for 30 min. 1 ml of spore of Inoculums of *trichoderma reesei* was inoculated in each conical flask. All flasks were kept in incubator at 30°C for 7 days. All flasks were shaken twice daily. Now these samples were used for DNS test for total reducing sugar.

DNS Test for Reducing Sugar

Reducing sugar was estimated by DNS method using DNS reagent (Miller 1959). After applying optimum conditions, treated and untreated hydrolysate were centrifuged at 13500 rpm for 20 min and supernatants obtained from centrifugation were used as crude extract for reducing sugar. 3ml of DNS reagent was added in each sample tube containing centrifuged samples and all tubes were incubated in water bath for 10 min to develop red brown color. All test tubes were taken out from water bath and cooled then added 1ml of 1% Rochella salt. All test tubes were left at room temperature for 20 min to established red brown color. Optical density was recorded by spectrophotometer at 540nm and compared with standard curve of glucose. Absorbance was compared with the standard graph plotted by reacting known concentration of glucose (.05 to 0.1mg/ml) with DNS reagent and plotting a graph between concentration of glucose (X axis) and OD at 540nm (Y axis).

RESULTS AND DISCUSSIONS

In present study *Eichhornia* plant was used as raw material for production of reducing sugar because this is found abundantly in ponds as a waste material. It does not play an important role in agricultural food for animals and its cost is very low. Out of selected many fungal species *Trichoderma reesei*, were observed as cellulose degrading microfungi to degrade cellulose and then reducing sugar was produced.

Effect of Incubation Period on Reducing Sugar Production

Reducing sugar production was determined at incubation period. It was seen that the hydrolysate of *Eichhornia crassipes* gave the higher sugar production which is 547µg/ ml in untreated samples and 596µg/ml in treated samples after 7th day of incubation. And the minimum production was 310 µg/ml, in untreated samples and 395 µg/ml in treated samples, were obtained after 3rd day of incubation. According to Ghose, (1987), hydrolysis rates decline with time due to depletion of the more amorphous substrates, product inhibition and enzyme inactivation. After optimum condition minimum production of reducing sugar highlights sugar depletion from substrate into the medium (Brien and Craig, 1996). Concentration of reducing sugar was found maximum in treated in compression to untreated samples in all optimized condition. So we can say acidic condition is better for reducing sugar production then non acidic condition.

Effect of pH on Reducing Sugar Production

Effect of pH on reducing sugar production was determined. It was observed that pH 3 is proved best in untreated hydrolysate and pH 5 in treated hydrolysate. 367µg/ml reducing sugar was produced at pH 3 in untreated and 521µg/ml reducing sugar at pH 5 in treated samples. Hydrolysate gave minimum reducing sugar at pH 7 that is 129 µg/ml, in untreated and 305 µg/ml, in treated samples. Effect of pH on reducing production from the untreated and treated hydrolysate by *T. reesei* was shown on Table no 2. Effect of pH on reducing sugar production was shown in Table no 2 which supports the findings of Lee *et al,* (2002), in which, pH optimum of β glucosidase was between 5 to 6. Microbial strains strongly depend on the extracellular pH because pH of the culture medium strongly influences many enzymatic processes and transport of various components across the cell growth and product production (Ellaiah *et. al*, 2002).

Effect of Temperature on Reducing Sugar Production

Optimum temperature for reducing sugar production was determined. It was observed that 30^0C temperature is best at which highest reducing sugar was produced. Hydrolysate gave higher reducing sugar production at 30^0C which is 458µg/ml in untreated and 593µg/ml in treated hydrolysate. It was observed minimum at 20^0C that is 289µg/ml and 382µg/ml respectively. Results of present study showed in Table no-3. Many researcher reported different temperatures for highest sugar production in flask using *Trichoderma sp*. It was suggested that the optimum temperature for sugar production also depends on micro fungi strains Suto and Tomito, (2001), Lu *et al*, (2003).

Effect of Substrate Concentration on Reducing Sugar Production

0.5% to 5 % of substrate concentrations were considered for production of reducing sugar. It was observed that the hydrolysate produced maximum reducing sugar production at 2.5% in untreated and at 2% in treated sample that are 373µg/ml and 507µg/ml respectively. Effect of substrate concentration for reducing sugar was shown in Table no 4. This supports the finding of Haapela *et al,* (1995) and Jeffries, (1996) who reported that if the substrate concentration increased after optimum level for glucose production did not found in appropriate increase in glucose yield. Reducing sugar production is increased till the availability of cellulose and the optimum concentration of sugar production began to decrease by inhibitory effect of accumulated cellobiose of low degree of polymerisation, of growth medium. It is done due to specific binding of the enzyme with substrates (Gilkes, *et al*, 1984).

ACKNOWLEDGEMENTS

The authors are very thankful to the UGC, New Delhi, for financial support and also grateful to Principal, Bipin Bihari PG Degree College, and Jhansi for constant support, encouragement and permission to do this research work.

Figures

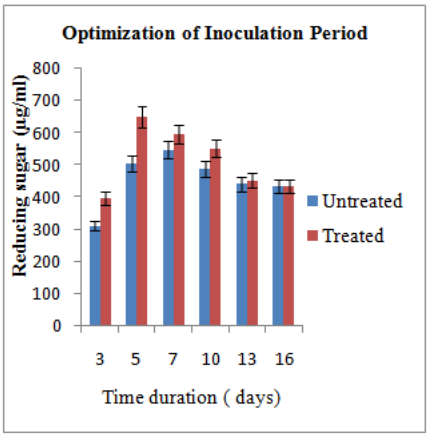

Figure 1: Effect of Inoculation Period on Reducing Sugar Production

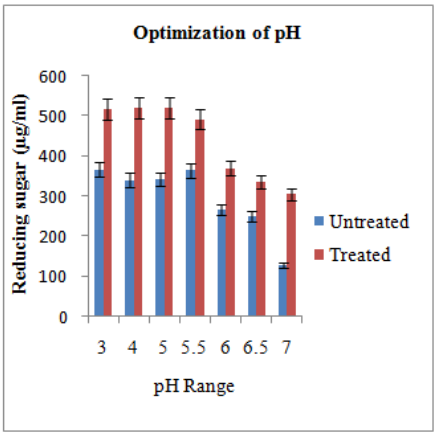

Figure 2: Effect of pH on Reducing Sugar Production

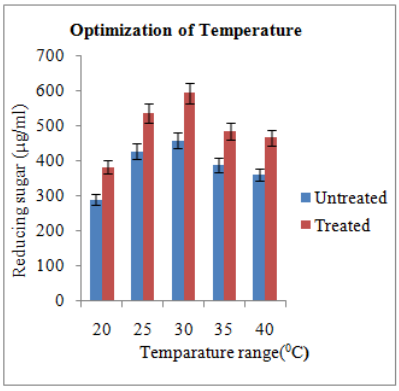

Figure 3: Effect of Temperature on Reducing Sugar Production

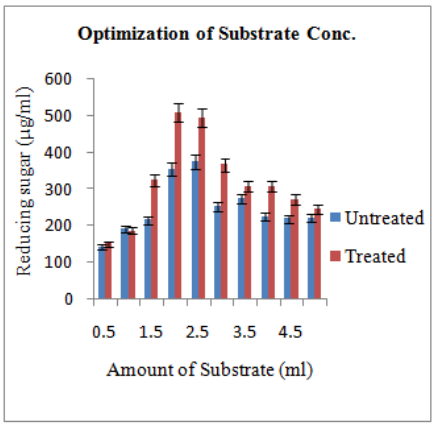

Figure 4: Effect of Substrate Concentration on Reducing Sugar Production

Tables

Table 1: Effect of Inoculation Period for Production of Reducing Sugar by *Trichoderma Reesei*

Inoculation Period	3rd Day	5th Day	7th Day	10th Day	13th Day	16th Day
(Untreated) Reducing sugar (µg/ml)	310	505	547	486	440	433
(Treated) Reducing sugar (µg/ml)	395	650	596	550	452	434

Table 2: Effect of Ph on Reducing Sugar Production by *Trichoderma Reesei*

pH	*3.0*	*4.0*	*5.0*	*5.5*	*6.0*	*6.5*	7.0
(Untreated) Reducing sugar (µg/ml)	367	341	342	365	268	250	129
(Treated) Reducing sugar (µg/ml)	518	520	521	492	370	335	305

Table 3: Effect of Temperature on Reducing Sugar Production of *Trichoderma Reesei*

(Tm)	20^0C	25^0C	30^0C	35^0C	40^0C
(Untreated) Reducing sugar (µg/ml)	289	427	458	387	360
(Treated) Reducing sugar (µg/ml)	382	536	593	483	465

Table 4: Effect of Substrate Concentration on Reducing Sugar Production by *Trichoderma Reesei*

Substrate Conc. (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.0	5.0
(Untreated) Reducing sugar(µg/ml)	140	190	215	352	373	250	273	223	218	220
(Treated) Reducing sugar(µg/ml)	150	185	322	507	492	365	307	307	271	244

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